LAB Research Ltd.

Address: 8200 Veszprém, Szabadságpuszta

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FINAL REPORT

SKIN SENSITIZATION:
LOCAL LYMPH NODE ASSAY
WITH [redacted]

Date of Final Report: 12 April 2010

STUDY CODE: 10/052-037E

STATEMENT OF THE STUDY DIRECTOR

This study has been performed in accordance with the study plan, the OECD Guidelines for Testing of Chemicals No.: 429. Skin Sensitization: Local Lymph Node Assay. (Adopted: 24th April 2002) and the Principles of Good Laboratory Practice (Hungarian GLP Regulations: 9/2001. (III. 30.) EüM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17.).

I, the undersigned, declare that this report constitutes a true record of the actions undertaken and the results obtained in the course of this study. By virtue of my dated signature I accept the responsibility for the validity of the data and the following conclusion drawn from them:

In conclusion, under the conditions of the present assay [redacted] tested in a suitable vehicle, was shown to have no sensitization potential (non-sensitizer) in the Local Lymph Node Assay.

Signature: Dr. Török Magdolna Date: 2 April 2010

Magdolna Török-Bathó, M.Sc.
Study Director

STATEMENT OF THE MANAGEMENT

According to the conditions of the research and development agreement between [redacted] Co., Ltd. (as Sponsor) and LAB Research Ltd. the study titled "Skin Sensitization: Local Lymph Node Assay with [redacted]" has been performed on CBA/J Rj mice, in accordance with the GLP requirements.

Signature: _____ Date: 12 Apr 2010

David J. Esdaile, M.Sc.
Scientific Director

QUALITY ASSURANCE STATEMENT

Study Code: 10/052-037E

Study Title: Skin Sensitization: Local Lymph Node Assay with [redacted]

This study has been inspected, and this report audited by the Quality Assurance Unit in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in written form to the study director and to management. The dates of such inspections and of the report audit are given below:

Date of Inspection	Phase(s) Inspected/Audited	Date of report to	
		Management	Study Director
23 February 2010	Study Plan	23 February 2010	23 February 2010
22 March 2010	^{3}HTdR solution injection	22 March 2010	22 March 2010
30 March 2010	Draft Report	30 March 2010	30 March 2010
12 April 2010	Final Report	12 April 2010	12 April 2010

Signature: Fábián Makovi Éva Date: 12 April 2010

Éva Makovi-Fábián, B.Sc.
On behalf of QA

STUDY TITLE : Skin Sensitization: Local Lymph Node Assay with [redacted]

TEST ITEM [redacted]

SPONSOR

STUDY PERFORMED BY : LAB Research Ltd.

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Tamás Mészáros, PhD – central dispensary, in reporting phase
Ágnes Móricz – statistical data processing
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Petra Tóth-Horváth, M.Sc. – quality assurance unit
Ágnes Rédl, M.Sc. – quality assurance unit
Ramóna Grób Heiderné, B.Sc. – quality assurance unit
Éva Makovi-Fábián, B.Sc. – quality assurance unit
Vanda Gyimesi, M.Sc. – quality assurance unit
Istvánné Kiss, M.Sc. – quality assurance unit

START OF EXPERIMENT : 17 March 2010
END OF EXPERIMENT : 23 March 2010
DATE OF DRAFT REPORT : 31 March 2010

BASIS OF STUDY : OECD Guidelines for Testing of Chemicals No. 429. Skin Sensitisation: Local Lymph Node Assay. Adopted: 24th April 2002

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1. SUMMARY

The aim of this study was to determine the skin sensitization potential of [redacted] following dermal exposure.

Based on the results of the Preliminary Compatibility Test and on the recommendations of the OECD Guideline [1], the test item was dissolved in acetone/olive oil 4:1 (v/v) mixture (AOO). The maximum attainable concentration was greater than 50 (w/v) %.

The Preliminary Irritation/Toxicity Test was performed in CBA/J Rj mice using two doses (test item concentrations of 50 and 25 (w/v) %) in the selected vehicle. The applicability and biocompatibility of the test item on the ears of animals at the maximum concentration of test item of 50 (w/v) % was acceptable.

In the main assay, sixteen female CBA/J Rj mice were allocated to four groups of four animals each:

- three groups received the appropriate formulation of [redacted] at concentrations of 50 %, 25 % and 10 (w/v) %,
- the negative control group received AOO.

The test item solutions were applied on the dorsal surface of ears of experimental animals (25 µl/ear) for three consecutive days (Days 1, 2 and 3). There was no treatment on Days 4, 5 and 6. On Day 6, the cell proliferation in the local lymph nodes was measured by incorporation of tritiated methyl thymidine (^{3}HTdR) and the values obtained were used to calculate stimulation indices (SI).

No mortality or systemic clinical signs were observed during the study. No treatment related effects were observed on animal body weights in any treated groups. The observed clinical signs are summarized in Appendix 4.

Stimulation index values of the test item were 1.6, 1.5 and 1.2 at treatment concentrations of 50 %, 25 % and 10 (w/v) %, respectively.

The result of the latest reliability check (performed within an interval of no longer than six months, Study code: 09/188-037E) was used to demonstrate the appropriate performance of the assay in accordance with the OECD guideline 429 [1]. The positive control substance α-Hexylcinnamaldehyde (HCA) was examined at a concentration of 25 % in the relevant vehicle. A significant lymphoproliferative response (SI ≥ 3) was noted for HCA with stimulation index value of 4.9, the result confirms the validity of the LLNA in this laboratory.

In conclusion, under the conditions of the present assay [redacted], tested in a suitable vehicle, was shown to have no sensitization potential (non-sensitizer) in the Local Lymph Node Assay.

2. INTRODUCTION

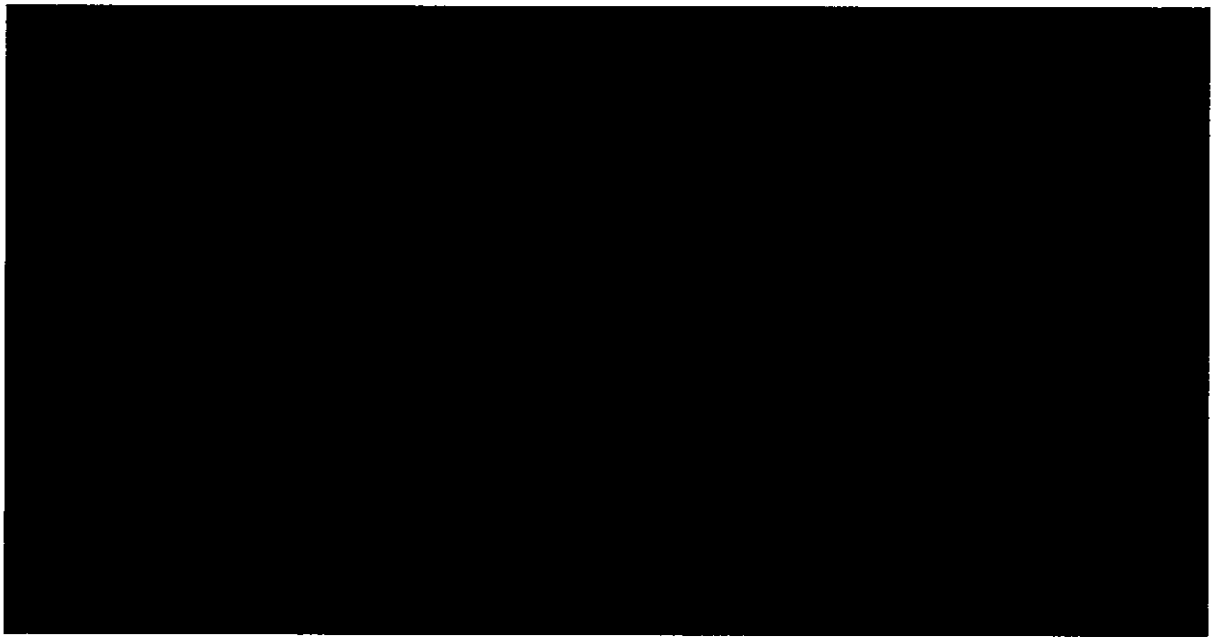
The basic principle underlying the LLNA is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application.

Generally, under appropriate test conditions, this proliferation is proportional to the concentration applied, and provides a means of obtaining an objective, quantitative measurement of sensitisation potential. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation into the DNA of dividing lymphocytes. The LLNA assesses proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in the vehicle treated control. The ratio of the proliferation in test groups to that in the control, termed Stimulation Index (SI), is determined and must be at least equal or greater than three, for a test substance to classify as a potential skin sensitizer.

The purpose of this study was to determine the skin sensitization potential of the test item following dermal exposure in the Local Lymph Node Assay.

3. MATERIALS AND METHODS

3.1. TEST ITEM



Any remaining test substance will be disposed after finalisation of all studies with this compound or 3 months after sending the (draft) reports.

3.1.1. Identification, Receipt

The test item of a suitable chemical purity was provided by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor. Identification of the test item was performed in the Central Dispensary Unit of LAB Research Ltd. on the basis of name, batch number, appearance and colour.

3.1.2. Formulation

During the Preliminary Compatibility Test the solubility of the test item was examined in Acetone: Olive oil 4:1 mixture (AOO). Since the solubility was greater than 50 % (w/v) in AOO, it was used as vehicle. The test item was weighed and formulations prepared daily on a weight: volume basis in the Central Dispensary Unit of LAB Research Ltd.

3.2. CONTROLS

3.2.1. Negative Control

Based on the results of the Preliminary Compatibility Test, Acetone: Olive oil 4:1 (v/v) mixture was chosen as vehicle for the Study. The abbreviation used for the vehicle in the Study Report is AOO.

Materials used for the preparation of the vehicle:

Name:	Acetone
Batch No.:	KBM59904
Manufacturer:	Reanal Co.
Expiry:	September 2014
Storage condition:	Room temperature

Name:	Olive oil
Batch No.:	058K0684
Manufacturer:	Sigma-Aldrich Co.
Expiry:	30 July 2010
Storage condition:	Room temperature

3.2.2. Positive Control

The results of the latest reliability check (performed using the positive control substance α-Hexylcinnamaldehyde (HCA) with the same vehicle (extractant substance) within an interval of no longer than 6 months) is used to demonstrate the appropriate performance of the assay.

The relevant dates for the latest reliability check (LAB Study Code: 09/188-037E) are as follows:

Start of Experimental phase:	16 September 2009
End of Experimental phase:	20 October 2009
Final Report:	04 November 2009

The data of the positive control substance used in the latest reliability check:

Name:	α-Hexylcinnamaldehyde, technical grade
Abbreviation:	HCA
CAS Number:	101-86-0
Lot No. :	02002DH
Manufacturer:	Sigma-Aldrich Co.
Nominal purity:	85 %
Purity:	99 %
Expiry:	30 December 2009
Storage condition:	Room temperature, 15 – 25 °C
Safety precautions:	Routine safety precautions (gloves, mask, lab coat, safety glasses) were applied to assure personnel health and safety.

3.3. OTHER CHEMICALS USED IN THE STUDY

The chemicals used are summarized in the following table:

Table 1: Chemicals Used in the Experiments

Chemical	Supplier	Batch Number	Expiry date
Distilled water	TEVA Co.	5001008	October 2011
Phosphate Buffered Saline, 10X Concentrate	Sigma-Aldrich Co.	039K8420	30 January 2011
Trichloroacetic acid (abbreviation: TCA)	Sigma-Aldrich Co.	039K1648	30 December 2010
[Methyl-3-H]-Thymidine	ARC Inc.	PP 010363 F / 100205	-
Optiphase HiSafe 3	PerkinElmer	152-091001	01 May 2011

3.4. INSTRUMENT SYSTEM

Name:	Tri-Carb 2810 Liquid Scintillation Analyzer
Serial Number:	DG10084483
IQ / OQ Protocol #:	1593646-1
Date of IQ / OQ:	25 November 2008
Manufacturer:	PerkinElmer

3.5. EXPERIMENTAL ANIMALS

Species and strain:	CBA/J Rj mice
Source:	ELEVAGE JANVIER Route des Chènes Secs B.P. 4105 53940 LE GENEST-ST-ISLE, France
Hygienic level at arrival:	SPF
Hygienic level during the study:	Standard housing conditions
Justification of strain:	On the basis of OECD Guideline, mice of CBA/Ca or CBA/J strain can be used. Females are used because the existing database is predominantly based on females.
Number of animals:	4 animals / treatment group
Sex:	female, nulliparous, non pregnant
Age of animals at starting:	11 – 12 weeks old
Body weight range at starting:	23.6 – 25.7 grams (The weight variation in animals involved in the study did not exceed ± 20 % of the mean weight)
Acclimatization time:	41 days

3.5.1. Husbandry

Animal health:	Only healthy animals were used for the study. Health status was certified by the veterinarian.
Housing / Enrichment:	Individual caging / mice were provided with glass tunnel-tubes
Cage type:	Type II. polypropylene/ polycarbonate
Bedding:	Bedding was available to animals during the study
Light:	12 hours daily, from 6.00 a.m. to 6.00 p.m.
Temperature:	22 ± 3 °C
Relative humidity:	30 - 70 %
Ventilation:	15-20 air exchange/hour

The temperature and relative humidity were recorded twice every day during the acclimatisation and experimental phases.

Room/Cabinet (non-radioactive phase): 244/6
Room/Cabinet (radioactive phase): 139 - 140

3.5.2. Food and feeding

Animals received ssniff SM R/M-Z+H "Autoclavable complete diet for rats and mice – breeding and maintenance" (Batch number: 476 3413 Expiry Date: June 2010) produced by ssniff Spezialdiäten GmbH (Ferdinand-Gabriel-Weg 16, D-59494 Soest, Germany), *ad libitum*. The contents of the standard diet are detailed in Appendix 2.

3.5.3. Water supply

Animals received tap water from the municipal supply from 500 ml bottle, *ad libitum*. Water quality control analysis was performed once every three months and microbiological assessment was performed monthly, by Veszprém County Institute of State Public Health and Medical Officer Service (ÁNTSZ, H-8201 Veszprém, József A.u.36., Hungary). Copies of the relevant Certificates of Analysis are retained in the Archive at LAB Research Ltd.

3.5.4. Bedding

Lignocel® Hygienic Animal Bedding produced by J. Rettenmaier & Söhne GmbH+Co.KG (D-73494 Rosenberger (Germany) Holzmühle 1) was available to animals during the study.

3.5.5. Identification

A unique number written on the tail with a permanent marker identified each animal. The animal number was assigned on the basis of LAB Research Ltd.'s master file. The cages were marked with identity cards with information including study code, cage number, and dose group, sex and individual animal number. The animals were randomised and allocated to the experimental groups. The randomisation was checked by computer software according to the actual body weights, verifying the homogeneity and variability between the groups.

3.6. ADMINISTRATION OF THE TEST ITEM

3.6.1. Dose Selection and Justification of Dose Selection

The Preliminary Irritation/Toxicity Test was performed in CBA/J mice using two doses (test item concentrations of 50 and 25 % (w/v)). This preliminary experiment was conducted in a similar experimental manner to the main study, but it was terminated on Day 6 with a body weight measurement (radioactive proliferation assay was not performed).

During the Preliminary Irritation/Toxicity Test no mortality was observed in any treatment groups. No significant body weight loss was observed in the treated groups. The observed clinical signs are summarized in Appendix 3.

The experimental groups and dose levels are summarized in Table 2.

Table 2: Experimental Groups and Treatments.

Groups	Test item concentration (% w/v)	No. of animals
Vehicle control (AOO)	-	4
[redacted]	50	4
	25	4
	10	4

3.6.2. Topical application

During the assay each mouse was topically dosed on the dorsal surface of each ear with 25 µl of the appropriate formulation applied using a pipette. Each animal was dosed once a day for three consecutive days (Days 1, 2 and 3). There was no treatment on Days 4, 5 and 6.

3.7. PROLIFERATION ASSAY

3.7.1. Injection of Tritiated Thymidine (^{3}HTdR)

On Day 6, animals were taken to the radioactive suite and each mouse was intravenously injected via the tail vein with 250 µl of sterile PBS (phosphate buffered saline) containing approximately 20 µCi of ^{3}HTdR using a gauge 25G1" hypodermic needle with 1 ml sterile syringe. Once injected, the mice were left for 5 hours (± 30 minutes).

3.7.2. Removal and Preparation of Draining Auricular Lymph Nodes

Five hours (± 30 minutes) after intravenous injection the mice were euthanized by asphyxiation with ascending doses of carbon dioxide (deep anaesthesia was confirmed before making incision, death was confirmed before discarding carcasses). The draining auricular lymph nodes were excised by making a small incision on the skin between the jaw and sternum, pulling the skin gently back towards the ears and exposing the lymph nodes. The nodes were then removed using forceps. The carcasses were discarded after cervical dislocation or after cutting through major cervical blood vessels. Once removed, the nodes of mice from each test group was pooled and collected in separate Petri dishes containing a small amount (1-2 ml) of PBS to keep the nodes wet before processing.

3.7.3. Preparation of Single Cell Suspension of Lymph Node Cells

A single cell suspension (SCS) of pooled lymph node cells (LNCs) wase prepared and collected in disposable tubes by gentle mechanical disaggregating of the lymph nodes through a cell strainer using the plunger of a disposable syringe. The cell strainer was washed with PBS (up to 10 ml). Pooled LNCs were pelleted with a relative centrifugal force (RCF) of 190 x g (approximately) for 10 minutes at 4 °C. After centrifugation supernatants were discarded. Pellets were gently resuspended and 10 ml of PBS was added to the tubes. The washing step was repeated twice.

This procedure was repeated for each group of pooled lymph nodes.

3.7.4. Determination of Incorporated ^{3}HTdR

After the final wash, supernatant were removed leaving a small volume (<0.5 ml) of supernatant above each pellet. Each pellet was gently agitated before suspending the LNCs in 3 ml of 5% TCA (trichloroacetic acid) for precipitation of macromolecules. After incubation with 5% TCA at 2-8 °C overnight (approximately 18 hours) precipitate was recovered by centrifugation at 190 x g for 10 minutes, supernatants were removed and pellets were suspended in 1 ml of 5% TCA and dispersed using ultrasonic water bath. Each precipitate was transferred to a suitable sized scintillation vial with 10 ml of scintillation liquid and thoroughly mixed. The vials were loaded to a β-scintillation counter and ^{3}HTdR incorporation was measured for up to 10 minutes per sample.

The β-counter expresses the ^{3}HTdR incorporation as the number of radioactive disintegrations per minute (DPM). Similarly, background ^{3}HTdR levels were also measured in two 1 ml aliquots of 5% TCA.

3.8. OBSERVATIONS

3.8.1. Clinical Observations

During the study (Day 1 to Day 6) each animal was observed daily for any clinical signs, including local irritation and systemic toxicity. Clinical observations were performed twice a day (before and after treatments) on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Individual records were maintained.

3.8.2. Measurement of Body Weight

Individual body weights were recorded on Day 1 (beginning of the test) and on Day 6 (prior to ^{3}HTdR injection) with a precision of ± 0.1 g.

3.9. EVALUATION OF THE RESULTS

DPM was measured for each pooled group of nodes. The measured DPM values were corrected with the background DPM value ("DPM"). The average of the two measured DPM values of 5 (w/v) % TCA solutions was used as the background DPM value. The results were expressed as "DPN" (DPM divided by the number of lymph nodes) following the industry standard for data presentation. Stimulation index (SI = DPN value of a treated group divided by the DPN value of the negative control group) for each treatment group was also calculated.
A stimulation index of 3 or greater is an indication of a positive result.

3.9.1. Interpretation of Results

The test item is regarded as a sensitizer if both of the following criteria are fulfilled:

- That exposure to at least one concentration of the test item resulted in an incorporation of ^{3}HTdR at least 3-fold or greater than recorded in control mice, as indicated by the stimulation index.
- The data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

4. USE OF RADIOACTIVE MATERIALS

Use of radioactive materials was recorded in the appropriate register. Regular decontamination of the working area with a verification of decontamination was carried out. Radioactive waste materials were processed according to normal laboratory standards.

5. PERMISSION OF THE IACUC

The conduct of the study was permitted by Institutional Animal Care and Use Committee (IACUC) of LAB Research Ltd.
Date of IACUC approval: 23 February 2010

6. ARCHIVES

The study documents and samples:

- study plan and amendment,
- all raw data,
- sample of the test item,
- study report and any amendments,
- correspondence

will be stored in the archives of LAB Research Ltd., 8200 Veszprém-Szabadságpuszta, Hungary according to the Hungarian GLP regulation and to test facility SOPs.

After the retention time agreed with the Sponsor has elapsed, all the archived materials listed above will be offered to the Sponsor or retained for a further period if agreed by a contract. Otherwise the materials will be discarded.

7. DEVIATION FROM THE STUDY PLAN

There was no deviation from the Study Plan.

8. RESULTS AND DISCUSSION

8.1. CLINICAL OBSERVATION

No mortality or signs of systemic toxicity were observed during the study.

8.2. BODY WEIGHT MEASUREMENT

No treatment related effects were observed on animal body weights. Individual and mean body weights are given in Table 3.

Table 3: Individual Body Weights for all Animals with Group Means

Animal Number	Identity Number	Test Group Name	Initial Body Weight (g)	Terminal Body Weight (g)
4556	1	Negative control (vehicle):	25.2	23.0
4568	2	AOO	25.6	26.1
4582	3		24.7	25.3
4563	4		23.6	22.7
		Mean	**24.8**	**24.3**
4574	5	[redacted]	25.5	24.7
4584	6		24.4	23.0
4576	7		24.5	23.4
4559	8		23.9	24.3
			24.6	**23.9**
4567	9		25.4	25.5
4575	10		24.7	23.8
4566	11		24.0	22.0
4558	12		23.7	23.4
			24.5	**23.7**
4570	13		25.7	24.2
4578	14		25.0	23.1
4583	15		24.8	24.6
4577	16		23.7	21.3
			24.8	**23.3**

8.3. PROLIFERATION ASSAY

The results of the proliferation assay are summarized in Table 4 and Figure 1. Appearance of the lymph nodes was normal in the negative control group and in the test item treated groups.

Table 4: DPM, DPN and Stimulation Index Values for all Groups

Test Group Name	Measured DPM/group	DPM	No. of Node	DPN	Stimulation Index Values
Background (5 (w/v) % TCA)	34.5		-		
Negative control AOO	905	870.5	8	108.8	1.0
[redacted]	1427	1392.5	8	174.1	1.6
	1329	1294.5	8	161.8	1.5
	1099	1064.5	8	133.1	1.2

8.4. INTERPRETATION OF OBSERVATIONS

The test item was a clear, colourless liquid with characteristic (solvents) odor which was dissolved in AOO.
Since there were no confounding effects of irritation or systemic toxicity at the applied concentrations, the proliferation values obtained are considered to reflect the real potential of the test item to cause lymphoproliferation in the Local Lymph Node Assay. The lack of any positive result under these exaggerated test conditions is considered to be good evidence that [redacted] is not a sensitizer (Figure 1).

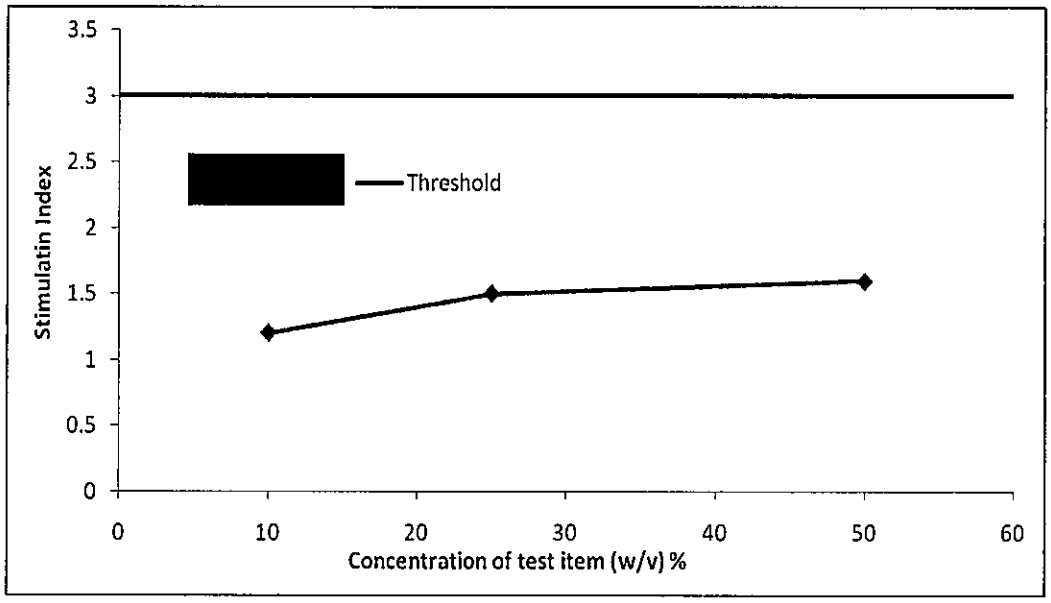


Figure 1. Test Item Stimulation Index Values

8.5. RELIABILITY OF THE TEST

The result of the latest reliability check (performed within an interval of no longer than six months) was used to demonstrate the appropriate performance of the assay in accordance with the OECD guideline [1]. The positive control substance α-Hexylcinnamaldehyde (HCA) was examined at a concentration of 25 % in the relevant vehicle (AOO) using CBA/J@Rj mice.

In the reliability check experiment, no mortality, cutaneous reactions or signs of toxicity were observed for the positive control substance. A significant lymphoproliferative response (SI ≥ 3) was noted for HCA dissolved in AOO with stimulation index value of 4.9. This value was considered to demonstrate the appropriate performance of the assay.

9. REFERENCES

1. OECD Guidelines for Testing of Chemicals No. 429. Skin Sensitisation: Local Lymph Node Assay. Adopted: 24th April 2002.
2. Hungarian GLP Regulations: 9/2001. (III.30.) EüM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development, which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17.

10. CONCLUSION

In conclusion, under the conditions of the present assay [redacted] tested in a suitable vehicle, was shown to have no sensitization potential (non-sensitizer) in the Local Lymph Node Assay.

APPENDICES

APPENDIX 1

STUDY SCHEDULE

	Relative Day	Absolute Day
PRE-EXPERIMENTAL PERIOD		
Animal receipt:	Day (-41)	04 February 2010
Veterinary control and acclimatisation:	from Day (-41) to Day 1	04 February 2010 17 March 2010
Animal identification:	Day 1	17 March 2010
Randomisation:	Day 1	17 March 2010
EXPERIMENTAL PERIOD		
Treatment days:	Day 1 Day 2 Day 3	17 March 2010 18 March 2010 19 March 2010
Body weight measurement:	Day 1 (beginning of the test) and Day 6 (prior to ^{3}HTdR injection)	17 March 2010 22 March 2010
Clinical observation:	daily from Day 1 to Day 6	17 March 2010 22 March 2010
Injection of ^{3}HTdR:	Day 6	22 March 2010
Preparation of LNC:	Day 6	22 March 2010
Sample measurement:	Day 7	23 March 2010
Date of Draft Report:		31 March 2010

APPENDIX 2

CONTENTS OF THE DIET

SSNIFF® SM R/M-Z+H COMPLETE DIET FOR RATS AND MICE

Batch No.: 476 3413 Best before: 06/2010

Crude Nutrients

Crude protein	19.00	%
Crude fat	3.50	%
Crude fiber	3.60	%
Crude ash	6.50	%
Calcium	1.00	%
Phosphorus	0.70	%
Sodium	0.20	%
Magnesium	0.22	%

Feed Additives

Vitamin A	25000	IU (per kg)
Vitamin D3	1000	IU (per kg)
Vitamin E	125	mg (per kg)
Copper,copper-(II)-sulfate pentahydrate	5	mg (per kg)
Lysine	1.10	%
Methionine	0.56	%

These data are standard and guaranteed values provided by the supplier.

APPENDIX 3

RESULT OF THE PRELIMINARY IRRITATION/TOXICITY TEST

Table 5: Individual Body Weights for all Animals with Group Means (Preliminary Irritation/Toxicity Test)

Animal Number	Identity Number	Test Group Name	Initial Body Weight (g)	Terminal Body Weight (g)*
4475	1	50 %	20.9	20.6
4485	2	50 %	21.4	20.4
		Mean:	**21.2**	**20.5**
4482	3	25 %	20.6	20.1
4488	4	25 %	21.8	20.1
		Mean:	**21.2**	**20.1**

*: Terminal body weights were measured on Day 6.

Table 6: Summarized Clinical Observations (Preliminary Irritation/Toxicity Test)

Period	Group	Identity No.	Animal No.	Clinical observations
DAY 1	50 % in AOO	1	4475	Before treatment: symptom-free After treatment: symptom-free
	50 % in AOO	2	4485	Before treatment: symptom-free After treatment: symptom-free
	25 % in AOO	3	4482	Before treatment: symptom-free After treatment: symptom-free
	25 % in AOO	4	4488	Before treatment: symptom-free After treatment: symptom-free
DAY 2	50 % in AOO	1	4475	Before treatment: alopecia After treatment: slightly rigid ear, alopecia
	50 % in AOO	2	4485	Before treatment: alopecia After treatment: slightly rigid ear, alopecia
	25 % in AOO	3	4482	Before treatment: symptom-free After treatment: symptom-free
	25 % in AOO	4	4488	Before treatment: symptom-free After treatment: symptom-free

Table 6 (Continued)

DAY 3	50 % in AOO	1	4475	Before treatment: area of alopecia was increasing After treatment: alopecia and erythema
	50 % in AOO	2	4485	Before treatment: area of alopecia was increasing After treatment: alopecia and erythema
	25 % in AOO	3	4482	Before treatment: minor alopecia After treatment: alopecia and erythema
	25 % in AOO	4	4488	Before treatment: minor alopecia After treatment: alopecia and erythema
DAY 4	50 % in AOO	1	4475	area of alopecia was increasing compared to Day 3
	50 % in AOO	2	4485	area of alopecia was increasing compared to Day 3
	25 % in AOO	3	4482	minor alopecia
	25 % in AOO	4	4488	minor alopecia
DAY 5	50 % in AOO	1	4475	area of alopecia was increasing compared to Day 4
	50 % in AOO	2	4485	area of alopecia was increasing compared to Day 4
	25 % in AOO	3	4482	area of alopecia same as Day 4
	25 % in AOO	4	4488	area of alopecia same as Day 4
DAY 6	50 % in AOO	1	4475	area of alopecia same as Day 5
	50 % in AOO	2	4485	area of alopecia same as Day 5
	25 % in AOO	3	4482	area of alopecia same as Day 5
	25 % in AOO	4	4488	area of alopecia same as Day 5

APPENDIX 4

SUMMARIZED CLINICAL OBSERVATIONS

Table 7: Summarized Clinical Observations

Group	Identity No.	Animal No.	CLINICAL OBSERVATIONS					
			DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
Neg. control (AOO)	1	4556	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	2	4568	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	3	4582	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	4	4563	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
[redacted]	5	4574	B. T.: symptom-free A. T: symptom-free	B. T.: alopecia A. T: slightly rigid ear , test item precipitate, alopecia	B. T.: slightly rigid ear , alopecia (the area was increasing) A. T: slightly rigid ear , test item precipitate, alopecia	slightly rigid ear , alopecia (the area was increasing)	alopecia	alopecia
	6	4584	B. T.: symptom-free A. T: symptom-free	B. T.: alopecia A. T: slightly rigid ear , test item precipitate, alopecia	B. T.: slightly rigid ear , alopecia (the area was increasing) A. T: slightly rigid ear , test item precipitate, alopecia	slightly rigid ear , alopecia (the area was increasing)	alopecia	alopecia
50 % (w/v)	7	4576	B. T.: symptom-free A. T: symptom-free	B. T.: alopecia A. T: slightly rigid ear , test item precipitate, alopecia	B. T.: slightly rigid ear , alopecia (the area was increasing) A. T: slightly rigid ear , test item precipitate, alopecia	slightly rigid ear , alopecia (the area was increasing)	alopecia	alopecia
	8	4559	B. T.: symptom-free A. T: symptom-free	B. T.: alopecia A. T: slightly rigid ear , test item precipitate, alopecia	B. T.: slightly rigid ear , alopecia (the area was increasing) A. T: slightly rigid ear , test item precipitate, alopecia	slightly rigid ear , alopecia (the area was increasing)	alopecia	alopecia

Table 7 (continued)

Group	Identity No.	Animal No.	CLINICAL OBSERVATIONS					
			DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
[redacted] 25 % (w/v)	9	4567	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	10	4575	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	11	4566	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	12	4558	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
[redacted] 10 % (w/v)	13	4570	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	14	4578	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	15	4583	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free
	16	4577	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	B. T.: symptom-free A. T: symptom-free	symptom-free	symptom-free	symptom-free

Note: B.T.: Before treatment; A.T.: After treatment;

APPENDIX 5

RESULTS OF THE LATEST RELIABILITY CHECK
(Study Code: 09/188-037E)

Table 8: DPM, DPN and Stimulation Index Values for all Groups of Reliability Check

Test Group Name	Measured DPM/group	DPM	No. of Node	DPN	Stimulation Index Values
Background (5 (w/v) % TCA)	31	-	-	-	-
Solvent Control: AOO	725.0	694.0	8	86.8	1.0
25% HCA in AOO	3421.0	3390.0	8	423.8	4.9
Solvent Control: DMF	929.0	898.0	8	112.3	1.0
25% HCA in DMF	10795.0	10764.0	8	1345.5	12.0
Solvent Control: DMSO	1102.0	1071.0	8	133.9	1.0
25% HCA in DMSO	8656.0	8625.0	8	1078.1	8.1
Solvent Control: 70% EtOH	485.0	454.0	8	56.8	1.0
25% HCA in 70% EtOH	17461.0	17430.0	8	2178.8	38.4
Solvent Control: HOO	981.0	950.0	8	118.8	1.0
25% HCA in HOO	4993.0	4962.0	8	620.3	5.2
Solvent Control: MEK	698.0	667.0	8	83.4	1.0
25% HCA in MEK	3332.0	3301.0	8	412.6	4.9
Background (5 (w/v) % TCA)	36	-	-	-	-
Solvent Control: PG	1905.0	1869.0	8	233.6	1.0
25% HCA in PG	9822.0	9786.0	8	1223.3	5.2
Solvent Control: 1% Pluronic	1192.0	1156.0	8	144.5	1.0
25% HCA in 1% Pluronic	5201.0	5165.0	8	645.6	4.5

APPENDIX 6

HISTORICAL CONTROL DATA

Table 9: Historical Control Data of the Positive Control Substance

	Solvents					
	Acetone- Olive oil (AOO)			1% Pluronic PE9200 in water (1%Plu)		
	DPN values		SI value	DPN values		SI value
	Control	HCA 25%	HCA 25%	Control	HCA 25%	HCA 25%
Average	237.7	1950.2	9.7	160.9	1347.4	9.3
Range: min	46.9	423.8	3.2	75.0	645.6	3.4
max	586.9	3300.5	28.5	469.6	2157.5	20.1
Number of cases	38	25	25	23	13	13

	Solvents								
	N,N-Dimethylformamide (DMF)			Dimethyl sulfoxide (DMSO)			n-Hexane:Olive oil (HOO)		
	DPN values		SI value	DPN values		SI value	DPN values		SI value
	Control	HCA 25%	HCA 25%	Control	HCA 25%	HCA 25%	Control	HCA 25%	HCA 25%
Average	186.8	2380.6	12.7	278.6	1933.8	8.1	124.4	1059.9	8.9
Range: min	39.0	1045.1	7.1	133.3	1052.8	4.2	81.1	490.0	5.0
max	423.1	4438.9	20.8	553.3	5291.3	24.1	165.9	1296.4	14.0
Number of cases	47	17	20	14	11	11	9.0	9.0	9.0

	Solvents							
	Propylene glycol (PG)			Absolute ethanol: Distilled water 70:30 mixture (EtOH)				
	DPN values		SI value	DPN values			SI values	
	Control	HCA 25%	HCA 25%	Control	HCA 10%	HCA 25%	HCA 10%	HCA 25%
Average	169.9	1569.9	7.5	137.2	1264.2	4041.9	17.3	32.4
Range: min	93.8	583.8	5.2	56.8	1214.8	2178.8	17.1	25.7
max	288.8	3231.3	11.2	357.6	1313.5	9207.1	17.4	38.4
Number of cases	10	5	5	5	2	4	2	4

HCA = alpha-Hexylcinnamaldehyde
SI (Stimulation Index) = DPN of a treated group divided by DPN of the appropriate control group.
DPN (Disintegrations Per Node) = DPM (Disintegrations Per Minute) divided by the number of lymph nodes.
In case of individual approach, SI values were calculated from the mean DPN values of the group.

APPENDIX 7

COPY OF GLP CERTIFICATE

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Budapest, 20th December 2008
No: 38625/48/2007
Our ref.: Szilvia Karsai
Subject: GLP Certificate

GOOD LABORATORY PRACTICE (GLP)
CERTIFICATE

Based on the Inspection report and the discussion of follow up activities it is hereby certified that the test facility

LAB Research Ltd.
H-8201 Veszprém, Szabadságpuszta, Hungary

is able to carry out **Physical-chemical testing, Toxicity studies, Mutagenicity studies, Environmental toxicity studies on aquatic and terrestrial organisms, Studies on behaviour in water, soil and air; bioaccumulation, Bioanalytical, Analytical and clinical chemistry testing** compliance with the Principles of GLP (Good Laboratory Practice).

Date of the inspection: **13-22 October 2008.**

This GLP Certificate is valid for 2 years.

Zsuzsanna Szepezdi, Ph. D.
Director-General